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An adenovirus-derived protein: A novel candidate for anti-diabetic drug development

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Abstract

Aims—Exposure to human adenovirus Ad36 is causatively and correlatively linked with better glycemic control in animals and humans, respectively. Although the anti-hyperglycemic property of Ad36 may offer some therapeutic potential, it is impractical to use an infectious agent for therapeutic benefit. Cell- based studies identified that Ad36 enhances cellular glucose disposal via its E4orf1 protein. Ability to improve glycemic control in vivo is a critical prerequisite for further investigating the therapeutic potential of E4orf1. Therefore, the aim of this study was to determine the ability of E4orf1 to improve glycemic control independent of insulin despite high fat diet.

Materials & Methods—8–9wk old male C57BL/6J mice fed a high-fat diet (60% kcal) were injected with a retrovirus plasmid expressing E4orf1, or a null vector (Control). Glycemic control was determined by glucose and insulin tolerance test. Islet cell size, amount of insulin and glucagon were determined in formalin-fixed pancreas. Rat insulinoma cell line (832/13) was infected with E4orf1 or control to determine changes in glucose stimulated insulin secretion. Protein from flash frozen adipose tissue depots, liver and muscle was used to determine molecular signaling by western blotting.

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Disclosure statement

NVD: The following Patents are granted or have been applied for:

A. United States Patents approved:

1. Number 6,127,113. Viral obesity methods and compositions.
2. Number 6,664,050. Viral obesity methods and compositions.
3. Number US 8,008,436B2: Adenovirus 36 E4orf1 gene & protein & their uses

B. Patents filed:

1. Adenovirus Ad36 E4orf1 protein for prevention and treatment of non-alcoholic fatty liver disease. US patent application 61/362,443; Taiwan Patent number 100124173.
2. Enhanced glycemic control using Ad36E4orf1 and AKT1 inhibitor

C. Provisional patent filed:

1. Compositions and methods for improving glucose uptake

He has ongoing grant support from Vital Health Interventions for determining anti-diabetic properties of E4orf1 protein.

Results—In multiple experiments, retrovirus-mediated E4orf1 expression in C57BL/6J mice significantly and reproducibly improved glucose excursion following a glucose load despite a high fat diet (60% energy). Importantly, E4orf1 improved glucose clearance without increasing insulin sensitivity, production or secretion, underscoring its insulin-independent effect. E4orf1 modulated molecular signaling in mice tissue, which included greater protein abundance of adiponectin, p-AKT and Glucose transporter Glu4.

Conclusions—This study provides the proof of concept for translational development of E4orf1 as a potential anti-diabetic agent. High fat intake and impaired insulin signaling are often associated with obesity, diabetes and insulin resistance. Hence, the ability of E4orf1 to improve glycemic control despite high fat diet and independent of insulin, is particularly attractive.

Keywords

Adenoviral protein; Glycemic control; Diabetes; Anti-diabetic drug; Glucose disposal; Insulin-independent effect

1. Introduction

Although about a dozen classes of anti-diabetes drugs are available, considerable need for better anti-diabetes drugs persists [1]. For example, high fat intake or impaired insulin signaling are often associated with diabetes. Yet, the current anti-diabetes drugs are insulin sensitizers, insulin mimetic or secretagogues, requiring insulin signaling for optimal benefits. Therefore, drugs that improve diabetes independent of dietary fat intake and insulin action may offer a significant advantage [2]. Here we describe such potential of the E4orf1 protein of human adenovirus Ad36, which may be translated to develop new anti-diabetic agents.

Microbes are a somewhat unconventional yet important source to develop therapeutic agents and strategies [3]. In animal models, Ad36 increases adiposity, but enhances glycemic control and reduces hepatic lipid accumulation, despite high fat diet and without recruiting the proximal insulin signaling [4–7]. Ad36 appears to enhance systemic glycemic control by promoting glucose uptake by adipose tissue and skeletal muscle, and by reducing hepatic glucose output and hepatic steatosis [8,9]. In rhesus monkeys, natural exposure to Ad36 is linked with a reduction in fasting glucose levels [10]. Interestingly, as cross-sectional and prospective associations, the key findings from Ad36-infected animals are mirrored in humans who are naturally exposed to Ad36 infection [4,11–22]. In humans, natural exposure to Ad36 is associated with obesity and fat gain, yet lower liver lipids and better glycemic control [4,11–22]. Natural Ad36 infection has also been shown to be associated with lower occurrence of type 2 diabetes with increased insulin sensitivity [23].

The potential of Ad36 to improve glycemic control is highly attractive for developing an effective anti-diabetic agent. Nonetheless, it is impractical to use a viral infection to improve diabetes. Instead, in vitro experiments identified that the E4orf1 protein, a 125 amino acid peptide [24], increases cellular glucose uptake in preadipocytes, adipocytes, and myoblasts, and reduces glucose output from hepatocytes [25,26]. These studies showed that E4orf1 bypassed the IRS mediated proximal insulin signaling and improved cellular glucose by up-

regulating the distal insulin signaling involving phosphatidyl insositol 3-kinase (PI3K), Akt and glucose transporter 4 (Glut4), via Ras activation [27]. These observations collectively showed that unlike the current anti-diabetic drugs, E4orf1 did not function as an insulin sensitizer, mimetic or secretagogue, but exhibited an insulin sparing effect [27]. This finding provided a single protein instead of an infectious agent, as a candidate to further investigate the anti-diabetic potential of Ad36 in vivo. Either the E4orf1 protein itself, or its analogs may be useful for developing therapeutic agent(s), provided, the protein improves glycemic control in vivo.

A proof of concept that E4orf1 protein enhances glycemic control in vivo is essential for further translating therapeutic properties of E4orf1. Hence, in multiple experiments, with the help of a retrovirus vector (pBabe), we expressed Ad36 *E4orf1* gene in high fat fed male C57BL/6J mice to determine the effect on glucose and insulin response and related cell signaling, and to assess the duration and reproducibility of the effect. As described below, the results demonstrated that E4orf1 is capable of improving glycemic control in vivo. This in vivo validation should stimulate further research towards developing E4orf1-based therapeutic agents.

2. Materials & Methods

2.1 Animal experiments

Institutional Animal Care and Use Committee (IACUC) of the Pennington Biomedical Research Center approved the protocols for animal studies. Mice were purchased from The Jackson Laboratories (Bar Harbour, Maine, USA) and placed on a 12 h light-dark cycle at 25 °C and housed in micro-isolator cages under Biosafety level-2 containment, with ad libitum access to food and water. End of study sacrifice was conducted by CO_2 asphyxiation followed by cervical dislocation. Based on a previous study [4] power analysis showed that a total of 6–9 mice were required in this parallel design study, at 90% probability to detect a treatment difference at a two-sided significance at 0.05.

Experiment 1: Does E4orf1 enhance blood glucose excursion in high fat fed mice?—Six-week old C57Bl/6J male mice on rodent chow (Purina Lab Diet 5001) were allowed 1-week of acclimatization following which total body fat was determined by Bruker Minispec mq10-NMR (Nuclear Magnetic Resonance) analyzer. Baseline GTT was performed as described under methods, to determine blood glucose clearance. The animals were then placed on a high fat (HF, 60% kcal) diet (Research Diets Inc. D12492i) for 2 weeks and GTT performed to determine HF diet induced hyperglycemia. Mice were divided into two groups (n = 9 each; Control or E4orf1) matched for body weight and inoculated with 300 μL pBabe-puro (Control) or pBabe-E4orf1 (10^8 copies of E4orf1) retrovirus as a combination of intra-peritoneal (i.p.), intra muscular (I.M.) and subcutaneous injections. GTT was performed 1 week post-infection (p.i.).

Experiment 2: Does E4orf1 transiently but reproducibly enhance glucose excursion?—Nine week old C57Bl/6J male mice on HF diet since 6 week of age were weight matched, divided into two groups and inoculated with pBabe-puro (Control; n = 6) or pBabe-E4orf1 (E4orf1; n = 6) retrovirus as described in Experiment 1. GTT was performed

1, 2, 3 and 4 week p.i. Mice were re-inoculated on week 7 p.i. and GTT performed 4 and 7 days post re-inoculation.

Experiment 3: Does a booster dose of E4orf1 extend the duration of enhancement in glucose clearance?—Eight week old C57Bl/6J male mice on HF diet since 6 wk of age, after 1 week of acclimatization and total body fat determination were weight matched into two groups. At 9 week of age the mice were inoculated with pBabe-puro (Control; n = 6) or pBabe-E4orf1 (E4orf1; n = 6) retrovirus as described in Experiment 1. GTT was performed 1 week p.i. and mice were re-inoculated at 10 week of age and GTT performed 1 week post re-inoculation to determine booster dose effect on blood glucose clearance. GTT was also performed 2 week post re-inoculation and 1 week post second booster inoculation.

Experiment 4: Does longer duration of high fat diet delay the improvement in GTT induced by E4orf1?—Eighteen C57Bl/6J male mice on HF diet since 6 week of age were weight matched, divided into three groups and total body fat content was determined. At 14 week of age the mice were inoculated with pBabe-puro (Control; n = 6) or pBabe-E4orf1 (E4orf1 300 μL; n = 6) or pBabe-E4orf1 (E4orf1 600 μL; n = 6) retrovirus as described in Experiment 1. GTT was performed 1, 2 and 3 week p.i. following which the mice were re-inoculated with a booster dose and GTT performed 4 days post re-inoculation.

Experiment 5: Does E4orf1 improve glucose clearance without increasing insulin sensitivity, production or secretion?—Nine week old C57Bl/6J male mice on HF diet since 6 week of age were weight matched, divided into two groups and inoculated with pBabe-puro (Control; n = 3) or pBabe-E4orf1 (E4orf1; n = 3) retrovirus as described in Experiment 1. Insulin tolerance test (ITT) was performed 1 and 2 week p.i. At the time of sacrifice (described in Experiment 6), trunk blood was collected and serum separated from fasted and non-fasted control (n = 3) and E4orf1 (n = 3) mice. Serum insulin was measured using insulin kit (Rat/Mouse Insulin ELISA, #EZRMI-13K, Millipore). Formalin fixed pancreas from control (n = 3) and E4orf1 (n = 3) were sectioned and beta cell mass was determined by selecting insulin expressing beta cell areas. These areas were then combined within a slice and the total area of insulin expressing cells was divided by the total tissue area on the slide and multiplied by 100. This gave the percent pancreas 2D section covered by beta-cells. The sectioned pancreata from multiple mice were also stained for insulin and glucagon to measure intensity between beta and alpha cells.

Glucose stimulated insulin secretion was determined in rat insulinoma 832/13 cells. Insulin was measured using the High Range Rat Insulin ELISA assay kit (Mercodia) as described in methods.

Experiment 6: Does E4orf1 enhance cell signaling in glucose disposal pathway?—Fasted (4 h) Control (n = 3) and E4orf1 (n = 3) mice were sacrificed by CO_2 asphyxiation and cervical dislocation and trunk blood was collected. Liver, muscle, pancreas, ingunal, epididymal, and retroperitoneal fat depots were carefully separated, weighed, flash frozen in liquid nitrogen, and stored at −80 °C. Protein lysates from tissues were used for western blot analysis to determine changes in protein expression of AKT, p-

AKT, AMPK, Glut4, adiponectin, Ras, PPARɣ, Glut2 and glucokinase in response to E4orf1 expression as described in methods.

2.2 Statistical analyses

Differences in glucose and insulin levels were analyzed by two-tailed student's 't' test for all animal experiments. Probability levels were set at $p \leq 0.05$. Groups for western blot analysis were compared using ANOVA followed by tukey's test.

2.3 Methods

2.3.1. Retrovirus—The human Adenovirus serotype 36 (Ad36) (from ATCC) early gene 4 open reading frame 1 (E4orf1) gene was amplified by PCR and cloned into the pBabe-puro retroviral vector (Cell Biolabs, Inc) at the BamHI-EcoRI site using restriction enzyme analysis. The constructed pBabe-E4orf1 and control pBabe-puro vectors were transformed into *E. coli* to make DNA stocks. The DNA was transfected into BOSC-23 cells (a gift from Dr. E. Floyd) to generate viral stocks and stored at −80 °C. BSL-2 safety measures were carried out during the viral stock generation and handling.

2.3.2. Glucose tolerance test—Subsequent to a 4-h fast, conscious mice were injected with D-glucose (1.5 mg/kg of body weight) intra-peritoneally. Blood was collected from the tail vein prior to glucose injection (time 0) and at 15, 30, 60, and 120 min post-injection. Blood glucose was deter- mined using a glucometer (Breeze contour, Bayer).

2.3.3. Insulin tolerance test—Mice were injected with insulin (0.75 U/kg of body weight) intra peritoneally after a 4-h fast. Blood glucose was measured using a glucometer at time 0, 15, 30, 60 and 90 min.

2.3.4. DNA extraction from pBabe retrovirus—DNA was extracted from pBabe-puro or -E4orf1 retrovirus stock using the viral DNA extraction kit (QIAamp MinElute Virus Spin kit, #57704, QIAGEN) following manufacturer's instructions.

2.3.5. Titration for pBabe retrovirus—The E4orf1 plasmid (100 ng/μL or 10^{10} copies/μL) was diluted 10 times to 10^{1} copies/μL. The extracted retroviral DNA from the pBabe-puro and pBabe-E4orf1 retrovirus stock was also diluted 10 times in DNase-free water. The samples or standard DNA (4 μL) was mixed with 2x TaqMan universal PCR Master Mix (#4304437, Applied Biosystems), primer and probe (PrimeTime Std qPCR Assay, #88953932, IDT) for E4orf1. The cDNA expression for E4orf1 was identified by quantative real-time PCR (ABI prism 7900HT). The titration of virus stock was calculated based on standard curve of E4orf1 plasmid.

2.3.6. ELISA for serum insulin—Serum from trunk blood was separated by centrifugation at 5000 rpm for 20 min and collected. Serum insulin was determined using a microtiter plate assay (Rat/Mouse Insulin ELISA, #EZRMI-13K, Millipore) as per manufacturer's instructions and the plates were read at 450 nm and 590 nm absorbance on a plate reader.

2.3.7. Western blot analyses—Proteins from adipose tissue, liver and skeletal muscle were used for western blot analyses by standard procedure previously described [4].

2.3.8. Glucose stimulated insulin secretion—832/13 cells are grown and passaged as described elsewhere [28]. For measurements of insulin secretion, cells were seeded into 12-well dishes, grown to 90% confluence and then treated with virus overnight in standard culture media. Insulin secretion assays were performed as described [28]. Insulin was measured using the High Range Rat Insulin ELISA assay kit (Mercodia). After collection of media for insulin analysis, the cells were lysed in M-PER lysis reagent (Thermo Scientifc) and BCA assays (Thermo Scientific) performed. Insulin secretion was normalized to total protein content.

2.3.9. Beta cell mass determination—Pancreas samples were processed and 4–5 μm sections were cut every 150 μm through the entire pancreas block. At each step-point, three serial sections were collected then 150 μm were removed and another three serial sections collected. Typically this resulted in 7–10 sections being sufficient to cover the entire pancreas thickness. The sections were stained on the Leica BOND system using rabbit anti-insulin antibody (Cell Signaling) at a 1:1000 dilution. The slides were dewaxed on the BOND and then subjected to a heat induced antigen retrieval using a low pH solution for 20 min followed by primary antibody incubation for 30 min and subsequent polymer-based detection (DAB). Slides were dehydrated, cover-slipped, and scanned on the Hamamatsu NanoZoomer for down-stream analysis in the NDPView software and ImageJ.

2.3.10. Insulin vs glucagon intensity—Slides were dewaxed prior to heat induced antigen retrieval with Biocare Rodent Decloaker at 100 °C for 15 min. After cooling for 30 min the slides were blocked for 1 h with goat serum block. Slides were incubated overnight at 4 °C with 1:400 dilutions of both Invitrogen guinea pig anti-insulin and Cell Signaling's rabbit anti-glucagon antibodies. After 17 h, the slides were washed in TBST prior to incubation with 1:300 dilutions of Invitrogen's goat anti-guinea pig Alexa 488 and Invitrogen's goat anti-rabbit Alexa 594 secondary antibodies for 1 h at RT. The slides were then washed in TBST prior to counterstaining with Hoechst.

After mounting and allowing the slides to cure for about 1–2 h, the slides were imaged on a Leica DM6000 microscope. Images were collected using a Hamamatsu EM-CCD (512 × 512 pixel) camera and were exported as single channel TIFF images for analysis in CellProfiler software. A CellProfiler pipeline was developed to identify insulin-positive beta cells as well as glucagon-positive alpha-cells based on green or red staining intensity, respectively. The area and integrated intensity of each cell type was collected and used to determine the average intensity of green (insulin) or red (glucagon) signal as well as the ratio of glucagon expressing vs insulin expressing cell area.

3. Results

3.1. E4orf1 enhances blood glucose excursion in high fat fed mice

pBabe vector expressed E4orf1 in mice tissue (Supplementary Fig. 1). Following an intraperitoneal glucose load, blood glucose excursion as determined by glucose tolerance

test (GTT) deteriorated when chow-fed 7-week old mice (n = 18) were switched to 60% (en %) high fat diet for two weeks (Fig. 1A). At this time, they were divided in two weight - matched groups (n = 9 each) and injected with either null virus vector (Control) or with virus vector expressing Ad36 E4orf1 (E4orf1). One week later, the E4orf1 group, but not the control group, significantly improved blood glucose clearance, as indicated by GTT and area under the curve (AUC) of GTT (Fig. 1B–E).

3.2. E4orf1 transiently but reproducibly enhances glucose excursion

Expression of E4orf1 in 9 week old mice on 60% fat diet, enhanced glucose excursion one week later, compared to that in control group of mice (Fig. 2A) and the effect persisted for one more week (Fig. 2B). The change in GTT due to E4orf1 expression was transient, as evident from GTT 3 and 4 week later which showed a lack of difference in GTT between the two groups (Fig. 2C & D). To test reproducibility of the effect, respective groups of mice were reinoculated with the null vector or E4orf1 expressing vector on week 7 after the first inoculation. As expected, the E4orf1 group improved GTT post re-inoculation (Fig. 2E & F).

3.3. A booster dose of E4orf1 does not extend the duration of enhancement in glucose clearance

Expression of E4orf1 in 9 week old mice on 60% fat diet, enhanced glucose clearance one week later, compared to that in control group of mice (Fig. 3A and B). A booster dose of respective vectors at this time did not extend the duration of enhancement in GTT by E4orf1, which continued till 2 week after the initial dose (Fig. 3C&D), but faded at 3 week post initial dose (Fig. 3E & F). A second booster given after the differences in GTT disappeared (Fig. 3E & F), again improved GTT for the E4orf group (Fig. 3G & H).

3.4. Longer duration of high fat diet delays the improvement in GTT induced by E4orf1

In mice on high fat diet for prior 8 weeks, E4orf1 expression did not improve GTT one week later (Fig. 4A & B). However, after a second dose of vectors after 3 weeks of the initial inoculation, E4orf1 significantly improved GTT (Fig. 4C & D). The improvement in GTT increased with the dose of E4orf1 vector (Fig. 4C & D).

3.5. E4orf1 improves glucose clearance without increasing insulin sensitivity, production or secretion

In high fat fed mice, E4orf1 enhanced glucose clearance 1 and 2 week later (Fig. 2 A & B), without an improvement in insulin sensitivity as determined by insulin tolerance test (ITT) (Fig. 5A–D). E4orf1 significantly reduced non-fasting insulin, but not the fasting insulin (Fig. 5E). E4orf1 does not appear to directly influence insulin production or secretion. Immuno-histology of mouse pancreatic islets showed no significant differences in alpha or beta cell areas or insulin or glucagon content in E4orf1 or Control groups (Tables 1 and 2). Rat insulinoma cells (832/13) infected with control or E4orf1 expressing pBabe retrovirus did not differ significantly from uninfected cells during assays measuring glucose stimulated insulin secretion (Fig. 5F).

3.6. E4orf1 enhances cell signaling in glucose disposal pathway

In vitro studies show that Ad36 E4orf1 up-regulates the Ras/PI3K/AKT/Glut4 pathway and adiponectin abundance, which likely promotes cellular glucose uptake [25,26]. The abundance of selected proteins in glucose disposal pathway was compared between the Control and E4orf1 groups in skeletal muscle, adipose tissue and liver. Compared to the Control group, E4orf1 significantly increased the abundance of p-AKT and glucose transporter Glut4 in skeletal muscle at the site of injection (right thigh) (Fig. 6 A & B). E4orf1 significantly increased the abundance of adiponectin protein in inguinal and retroperitoneal fat pads (Fig. 6C & D), and that of Ras in inguinal fat pad (Fig. 6C & D). No significant differences were detected in the protein abundance of Glut4, PPARγ, or p-AKT in fat pads of the two groups (Fig. 6C & D). Of the liver proteins compared, E4orf1 significantly influenced the abundance of only Glucose transporter 2 (Glut 2), but not p-AKT or p-AMPK or glucokinase (Fig. 6E).

4. Discussion

We first observed the anti-hyperglycemic effect of adenovirus 36 in rats and chow or high fat fed mice [4,5]. Subsequent in vitro experiments narrowed down the effect to E4orf1 protein of adenovirus 36. Therefore, to test the anti-hyperglycemic effect of E4orf1 in vivo, we used high fat fed mice, a model that resembles human diet-induced hyperglycemia. This mouse model improved glycemic control transiently but predictably and reproducibly, in response to E4orf1 booster doses. Recently, Kusminski et al. [29] also inducibly expressed E4orf1 in adipose tissue of high fat fed mice to improve glycemic control, indicating suitability of the model to further study the phenomenon.

Multiple separate experiments reproducibly demonstrated the potential of Ad36 E4orf1 protein to enhance blood glucose clearance in mice in presence of high fat diet. We determined E4orf1 protein expression in muscle and liver via PCR (Supplemental information). Considering that Ad36E4orf1 is a viral protein and administered exogenously, its expression is too low in tissues for detection by western blotting. Therefore we instead determined the presence of E4orf1 DNA in tissue by PCR as evidence of Ad36E4orf1 presence.

Collectively, these experiments indicate that the capability of Ad36 to improve glycemic control [4,5] can be successfully captured by expressing its E4orf1 protein in vivo. Although previous studies revealed that Ad36 E4orf1 is necessary and sufficient to enhance cellular glucose uptake in vitro [26], improvement by E4orf1 in glucose excursion in vivo after a glucose challenge is a pivotal finding. This provides the proof of concept for further development of E4orf1 as an anti-diabetic agent. Nonetheless, as discussed below, several aspects need to be considered before E4orf1 could be translated to a therapeutic use.

Much of the understanding of in vitro molecular interaction of E4orf1was initially based on the information about E4orf1 protein of human adenovirus 9 (Ad9), which bears a 92% sequence homology with E4orf1 of Ad36. E4orf1 proteins have a PDZ domain binding motif (PBM) at the C-terminal. Through its PBM, the peptide binds to PDZ proteins, which are scaffolding proteins that facilitate protein interactions. E4orf1 proteins of Ad9 as well as

Ad36 require their PBM for binding to Dlg (Drosophila disc large)-1 protein. After the binding in cytosol, the complex travels to the cell membrane, where it activates Ras and subsequently, PI3K [30–32]. Ad36 E4orf1requires intact PBM for Ras induction, and downstream activation of AKT signaling and glucose uptake [26].

As the first investigation of its kind, this study mapped out a number of fundamental attributes of the in vivo action of Ad36 E4orf1. A key feature is that the improvement in glycemic control is transient, yet reproducible, upon re-introduction of E4orf1. This indicates that probably, the effect is transient due to diminishing E4orf1 expression over 2 weeks, and not due to development of resistance to the action of E4orf1 itself. Although the retrovirus vector pBabe-puro used for E4orf1 expression should stably integrate in host DNA and provide a longer lasting expression of E4orf1, the gene expression and hence the effect of E4orf1 appears to last only a few days in vivo. In fact, the transient effect of E4orf1 on glucose clearance helps demonstrate the reproducibility of response of mice when exposed to E4orf1. Although this study was not designed to systematically evaluate E4orf1 dose response, a further decrease in AUC of GTT in response to a booster dose (Fig. 3D) or greater amount of vector (300 vs 600 μL) (Fig. 4B) suggested that the reduction in glucose excursion is related to E4orf1 dose.

Another important attribute is that E4orf1 appears to promote blood glucose clearance independent of insulin involvement. Although, enhanced sensitivity, production or release of insulin, or a decrease in insulin antagonism by glucagon are some key determinants of glucose excursion after a glucose load [33], E4orf1 did not modulate them (Fig. 5, Tables 1 and 2). Instead, it is proposed that the insulin requirement for controlling glucose disposal is reduced in presence of E4orf1, termed as the insulin sparing effect of E4orf1 [34]. This was also apparent from a reduction in non- fasting serum insulin levels in E4orf1 (Fig. 5E). Fasting serum insulin was not different between the E4orf1 expressing or control mice. However serum insulin was significantly lower in E4orf1 expressing animals under fed conditions. The combination of better glucose clearance and lower insulin levels suggests that these mice cleared post-prandial glucose increase without increasing insulin secretion. This could be considered as insulin sparing action of E4orf1. Improved glucose clearance as seen in this study is consistent with a decrease in circulating insulin because insulin resistance and insulin secretion are positively correlated. Thus, E4orf1 delivery appears to enhance glucose clearance in a manner analogous to exercise, i.e., by stimulating uptake into tissues such as skeletal muscle without requiring insulin.

Ad36 and E4orf1 protein bypass the proximal insulin signaling, and increase cellular glucose uptake independent of insulin stimulation, by Ras-mediated up-regulation of AKT and Glut4 signaling in cells of adipose tissue and skeletal muscle [26,34]. Moreover, Ad36 and E4orf1 reduce hepatic Glut2 protein abundance [4], which may contribute to the reduction in glucose output by hepatocytes [25]. Taken together, it is postulated that E4orf1 improves glycemic control by increasing skeletal muscle and adipose tissue glucose uptake, and by reducing hepatic glucose output, independent of insulin action. In accordance, we observed greater abundance of Ras, p-AKT, Glut4 and adiponectin, and a reduction in hepatic Glut2 (Fig. 6) in some, but not in all tissues examined. pBabe vectors were

introduced by intraperitoneal, intra-muscular and subcutaneous routes in select sites. This may have led to non-uniform tissue distribution of E4orf1 expression.

Additional in vivo studies are needed to further elucidate how E4orf1 modulates cellular insulin signaling and to identify specific tissue contribution to systemic glucose levels. Nonetheless, considering that diabetes is often linked with insulin resistance or impaired proximal insulin signaling [35], the insulin sparing property of E4orf1 is attractive. It is also interesting that E4orf1 improved glycemic control despite a very high fat intake. This is particularly important because excessive kcal intake from lipid sources is often linked with weight gain, insulin resistance and diabetes [36]. Although a reduction in dietary fat intake and weight loss are often recommended as adjunct to diabetes treatment, making such dietary changes is highly challenging for many individuals [37]. Hence, the ability of E4orf1 to improve glycemic control in presence of high fat diet is particularly valuable by providing options other than weight loss to control glycemia.

While this study provided critical impetus to harnessing the potential of Ad36 E4orf1 as a novel anti-diabetic agent in a model of diet-induced hyperglycemia, considerable research is needed to effectively exploit its therapeutic property. This includes a determination of the role of E4orf1 in preventing and treating diabetic in genetic models of obesity and diabetes, and in models of type 1 diabetes. Furthermore, a very careful evaluation of safety of long term exposure to E4orf1 is needed. Given the potential role of Ras in tumorogenesis [38], the in vivo effect of E4orf1 requires careful investigation. However, Ras activation is important for regular cell functioning and the role of Ras in tumorogenesis is mainly attributed to missense mutation leading to uncontrolled Ras expression [38]. Whereas, Ras overexpression was shown to improve glycemic control without any tumor formation [39]. No tumors were observed in previous experiments when animals were infected with Ad36. Nonetheless, the consequence of Ras modulation by E4orf1 [31] should be further evaluated. Finally, an effective approach is needed to therapeutically deliver E4orf1 or E4orf1 mimetic agents.

Conceptually, these findings open a research opportunity to develop a new anti-diabetic agent with multiple uniquely beneficial properties. This may lead to the translation of a rather unconventional source, microbial proteins, for therapeutic benefit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2015.12.002.

References

1. Rotenstein LS, Kozak BM, Shivers JP, Yarchoan M, Close J, Close KL. The ideal diabetes therapy: what will it look like? how close are we? Clin Diabetes. 2012; 30:44–53.
2. Kaiser D, Oetjen E. Something old, something new and something very old: drugs for treating type 2 diabetes. Br J Pharmacol. 2014; 171:2940–2950. [PubMed: 24641580]
3. Dhurandhar NV, Geurts L, Atkinson RL, Casteilla L, Clement K, Gerard P, Vijay-Kumar, Nam JH, Nieuwdorp M, Trovato G, Sorensen TI, Vidal-Puig A, Cani PD. Harnessing the beneficial properties of adipogenic microbes for improving human health. Obes Rev. 2013; 14:721–735. [PubMed: 23663746]
4. Krishnapuram R, Dhurandhar EJ, Dubuisson O, Kirk-Ballard H, Bajpeyi S, Butte, Sothern MS, Larsen-Meyer E, Chalew S, Bennett B, Gupta AK, Greenway FL, Johnson W, Brashear M, Reinhart G, Rankinen T, Bouchard C, Cefalu WT, Ye J, Javier R, Zuberi A, Dhurandhar NV. Template to improve glycemic control without reducing adiposity or dietary fat. Am J Physiol Endocrinol Metab. 2011; 300:E779–E789. [PubMed: 21266671]
5. Pasarica M, Shin AC, Yu M, Ou Yang HM, Rathod M, Jen KL, MohanKumar S, MohanKumar PS, Markward N, Dhurandhar NV. Human adenovirus 36 induces adiposity, increases insulin sensitivity, and alters hy-pothalamic monoamines in rats. Obes (Silver Spring). 2006; 14:1905–1913.
6. Dhurandhar NV, Whigham LD, Abbott DH, Schultz-Darken NJ, Israel BA, Bradley SM, Kemnitz JW, Allison DB, Atkinson RL. Human adenovirus Ad- 36 promotes weight gain in male rhesus and marmoset monkeys. J Nutr. 2002; 132:3155–3160. [PubMed: 12368411]
7. Na HN, Nam JH. Adenovirus 36 as an obesity agent maintains the obesity state by increasing MCP-1 and inducing inflammation. J Infect Dis. 2012; 205:914–922. [PubMed: 22275403]
8. Rogers PM, Mashtalir N, Rathod MA, Dubuisson O, Wang Z, Dasuri K, Babin S, Gupta A, Markward N, Cefalu WT, Dhurandhar NV. Metabolically favorable remodeling of human adipose tissue by human adenovirus type 36. Diabetes. 2008; 57:2321–2331. [PubMed: 18599527]
9. Wang ZQ, Cefalu WT, Zhang XH, Yu Y, Qin J, Son L, Rogers PM, Mashtalir N, Bordelon JR, Ye J, Dhurandhar NV. Human adenovirus type 36 enhances glucose uptake in diabetic and nondiabetic human skeletal muscle cells independent of insulin signaling. Diabetes. 2008; 57:1805–1813. [PubMed: 18420488]
10. Dhurandhar NV, Dhurandhar EJ, Ingram DK, Vaughan K, Mattison JA. Natural infection of human adenovirus 36 in rhesus monkeys is associated with a reduction in fasting glucose 36. J Diabetes. 2014:614–616. [PubMed: 24909894]
11. Dhurandhar NV. A framework for identification of infections that contribute to human obesity. Lancet Infect Dis. 2011; 11:963–969. [PubMed: 22115071]
12. Lin W, Dubuisson O, Rubicz R, Liu N, Allison DB, Curran JE, Comuzzie AG, Blangero J, Leach CT, Goring H, Dhurandhar NV. Long term changes in adiposity and glycemic control are associated with past adenovirus infection. Diabetes Care. 2013; 36:701–707. [PubMed: 23160725]
13. Atkinson RL, Dhurandhar NV, Allison DB, Bowen RL, Israel BA, Albu JB, Augustus AS. Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. Int J Obes (Lond). 2005; 29:281–286. [PubMed: 15611785]
14. Trovato GM, Castro A, Tonzuso A, Garozzo A, Martines GF, Pirri C, Trovato F, Catalano D. Human obesity relationship with Ad36 adenovirus and insulin resistance. Int J Obes (Lond). 2009; 33:1402–1409. [PubMed: 19786969]
15. Trovato GM, Martines GF, Garozzo A, Tonzuso A, Timpanaro R, Pirri C, Trovato FM, Catalano D. Ad36 adipogenic adenovirus in human non- alcoholic fatty liver disease. Liver Int. 2010; 30:184–190. [PubMed: 19840251]

16. Trovato GM, Martines GF, Trovato FM, Pirri C, Pace P, Garozzo A, Castro A, Catalano D. Adenovirus-36 seropositivity enhances effects of nutritional intervention on obesity, bright liver, and insulin resistance. Dig Dis Sci. 2012; 57:535–544. [PubMed: 21953137]
17. Na HN, Kim J, Lee HS, Shim KW, Kimm H, Jee SH, Jo I, Nam JH. Association of human adenovirus-36 in overweight Korean adults. Int J Obes (Lond). 2012; 36:281–285. [PubMed: 21587203]
18. Na HN, Hong YM, Kim J, Kim HK, Jo I, Nam JH. Association between human adenovirus-36 and lipid disorders in Korean schoolchildren. Int J Obes (Lond). 2010; 34:89–93. [PubMed: 19823186]
19. Atkinson RL, Lee I, Shin HJ, He J. Human adenovirus-36 antibody status is associated with obesity in children. Int J Pediatr Obes. 2009:1–4.
20. Gabbert C, Donohue M, Arnold J, Schwimmer JB. Adenovirus 36 and obesity in children and adolescents. Pediatrics. 2010; 126:721–726. [PubMed: 20855385]
21. Aldhoon-Hainerova I, Zamrazilova H, Atkinson RL, Dusatkova L, Sedlackova, Hlavaty P, Lee ZP, Kunesova M, Hainer V. Clinical and laboratory characteristics of 1179 Czech adolescents evaluated for antibodies to human adenovirus 36. Int J Obes Lond. 2014; 38:285–291. [PubMed: 23732656]
22. Almgren M, Atkinson R, He J, Hilding A, Hagman E, Wolk A, Thorell A, Marcus, Naslund E, Ostenson CG, Schalling M, Lavebratt C. Adenovirus-36 is associated with obesity in children and adults in Sweden as determined by rapid ELISA. PloS One. 2012; 7:e41652. [PubMed: 22848557]
23. Almgren M, Atkinson RL, Hilding A, He J, Brismar K, Schalling M, Ostenson CG, Lavebratt C. Human adenovirus-36 is uncommon in type 2 diabetes and is associated with increased insulin sensitivity in adults in Sweden. Ann Med. 2014; 46:539–546. [PubMed: 25045929]
24. Rogers PM, Fusinski KA, Rathod MA, Loiler SA, Pasarica M, Shaw MK, Kilroy G, Sutton GM, McAllister EJ, Mashtalir N, Gimble JM, Holland TC, Dhurandhar NV. Human adenovirus Ad-36 induces adipogenesis via its E4 orf-1 gene. Int J Obes (Lond). 2008; 32:397–406. [PubMed: 17984979]
25. Dhurandhar EJ, Krishnapuram R, Hegde V, Dubuisson O, Tao R, Dong XC, Ye J, Dhurandhar NV. E4orf1 improves lipid and glucose metabolism in hepatocytes: a template to improve steatosis & hyperglycemia. PloS One. 2012; 7:e47813. [PubMed: 23110104]
26. Dhurandhar EJ, Dubuisson O, Mashtalir N, Krishnapuram R, Hegde V, Dhurandhar NV. E4orf1: a novel ligand that improves glucose disposal in cell culture. PLoS ONE. 2011; 6(8):e23394. [PubMed: 21886789]
27. Dhurandhar NV. Insulin sparing action of adenovirus 36 and its E4orf1 protein. J Diabetes Complicat. 2013; 27:191–199. [PubMed: 23246247]
28. Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB. Isolation of INS-1-derived cell lines with robust ATP-sensitive Kþ channel-dependent and -independent glucose-stimulated insulin secretion. Diabetes. 2000; 49:424–430. [PubMed: 10868964]
29. Kusminski CM, Gallardo-Montejano VI, Wang ZV, Hegde V, Bickel PE, Dhurandhar NV, Scherer PE. E4orf1 induction in adipose tissue promotes insulin-independent signaling in the adipocyte. Mol Metab. 2015; 4:653–664. [PubMed: 26500839]
30. Frese KK, Lee SS, Thomas DL, Latorre IJ, Weiss RS, Glaunsinger BA, Javier RT. Selective PDZ protein-dependent stimulation of phosphatidylino- sitol 3-kinase by the adenovirus E4-ORF1 oncoprotein. Oncogene. 2003; 22:710–721. [PubMed: 12569363]
31. Kumar M, Kong K, Javier RT. Hijacking discs large 1 for oncogenic phos-phatidylinositol 3-kinase activation in human epithelial cells is a conserved mechanism of human adenovirus E4-ORF1 proteins. J Virol. 2014; 88:14268–14277. [PubMed: 25253337]
32. Kong K, Kumar M, Taruishi M, Javier RT. The human adenovirus E4-ORF1 protein subverts discs large 1 to mediate membrane recruitment and dysregulation of phosphatidylinositol 3-kinase. PLoS Pathog. 2014; 10:e1004102. [PubMed: 24788832]
33. Postprandial blood glucose. American diabetes association. Diabetes Care. 2001; 24:775–778. [PubMed: 11315848]
34. Dhurandhar NV. Insulin sparing action of Adenovirus 36 and its E4orf1 protein. J Diabetes Its Complicat. 2013; 27:191–199.

35. Frojdo S, Vidal H, Pirola L. Alterations of insulin signaling in type 2 diabetes: a review of the current evidence from humans. Biochim Biophys Acta. 2009; 1792:83–92. [PubMed: 19041393]

36. Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN. Diet- induced type II diabetes in C57BL/6J mice. Diabetes. 1988; 37:1163–1167. [PubMed: 3044882]

37. Dhurandhar NV. When commonsense does not make sense. Int J Obes. 2012; 36:1332–1333.

38. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. Nat Rev Cancer. 2011; 11:761–774. [PubMed: 21993244]

39. Houseknecht KL, Zhu AX, Gnudi L, Hamann A, Zierath JR, Tozzo E, Flier JS, Kahn BB. Overexpression of Ha-ras selectively in adipose tissue of transgenic mice. Evidence for enhanced sensitivity to insulin. J Biol Chem. 1996; 271:11347–11355. [PubMed: 8626688]

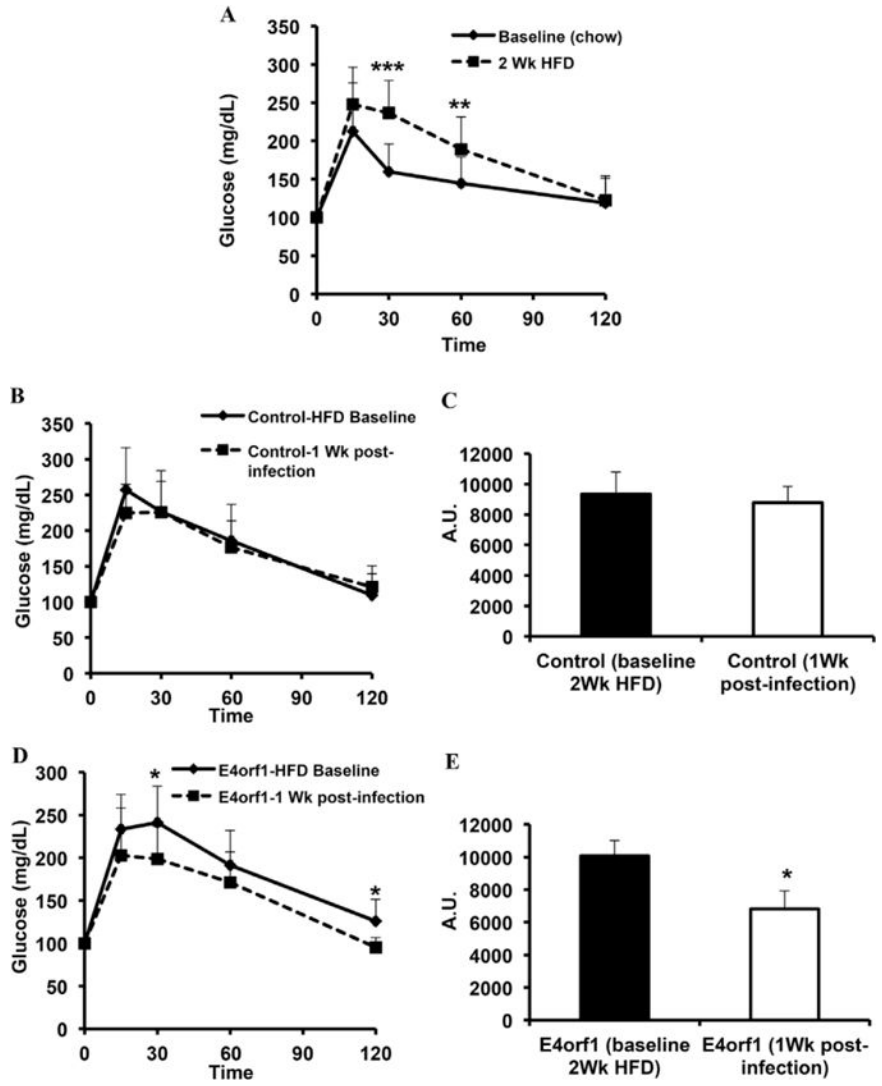

Fig. 1.

E4orf1 enhances blood glucose excursion in high fat fed mice: A. Eighteen, weight-matched 7 week old C57Bl/6J male mice on chow diet were divided into two groups (Control; n = 9 and E4orf1; n = 9) followed by baseline glucose tolerance test (GTT; black solid line). The mice were fed a 60% kcal high fat (HF) diet for 2 week and GTT performed (black dotted line). The animals were inoculated with pBabe retrovirus expressing control puro (B–C) or Ad36 E4orf1 (D–E) and GTT performed 1 week post inoculation. (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$). The bar graphs show area under the curve for respective groups.

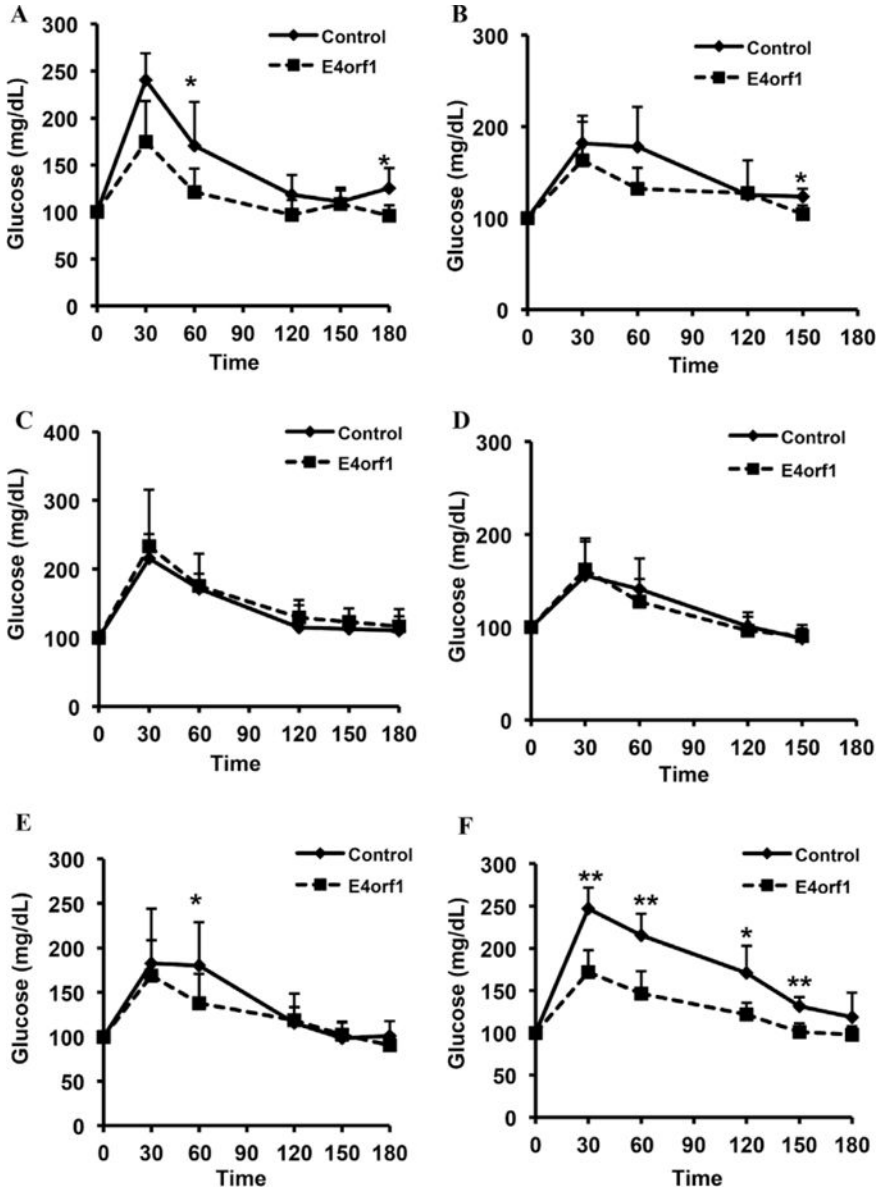

Fig. 2.

E4orf1 transiently but reproducibly enhances glucose excursion: C57BL/6J (9 week old) mice on 60% fat diet since 6 week of age were inoculated with pBabe-puro retrovirus (control; black solid lines, n = 6), or with pBabe expressing E4orf1 (black dotted lines; n = 6). Expression of E4orf1, enhanced blood glucose clearance 1 week later, compared to control group of mice (A) and the effect persisted for one more week (B). The effect of E4orf1 on GTT was transient and weeks 3 and 4 post-inoculation (p.i.) showed that the effect of first inoculation had faded (C–D). Upon re-inoculation in week7, GTT significantly improved again for the E4orf1 group 4 d (E) and a week later (F). (*$p < 0.05$, **$p < 0.005$).

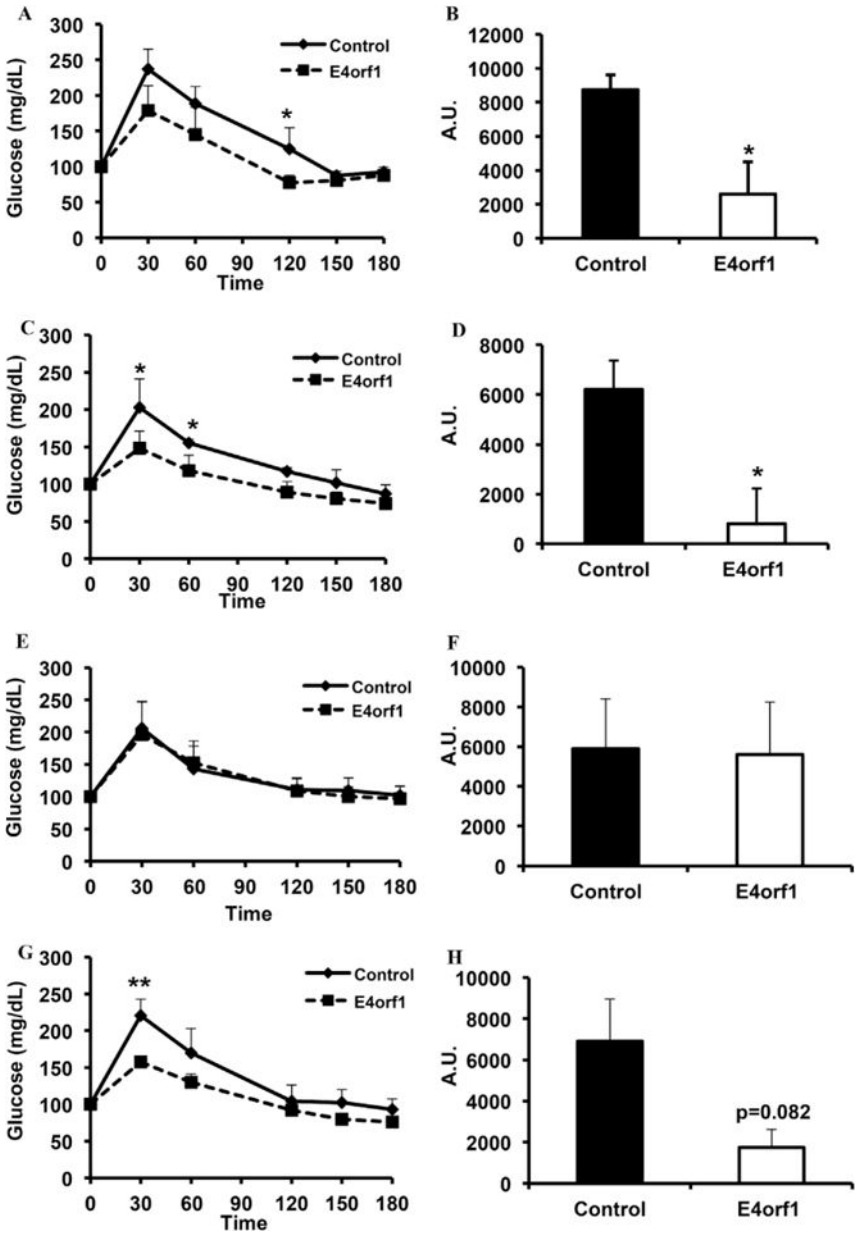

Fig. 3.

A booster dose of E4orf1 does not extend the duration of enhancement in glucose clearance: Nine week old C57BL/6J (9 wk old) male mice on 60% HF diet since 6 wk of age were inoculated with control retrovirus (black solid lines, n = 6), or with E4orf1 (black dotted lines; n = 6). GTT was performed 1 week p.i. (A–B) and mice were re-inoculated at 10 week of age and GTT performed 1 week post re-inoculation (C–D). The improved glucose clearance following each inoculation disappeared 3 week post initial inoculation (E–F) but a second booster inoculation again improved GTT for the E4orf1 group (G–H). (*$p < 0.05$, **$p < 0.005$). The bar graphs show area under the curve for respective groups.

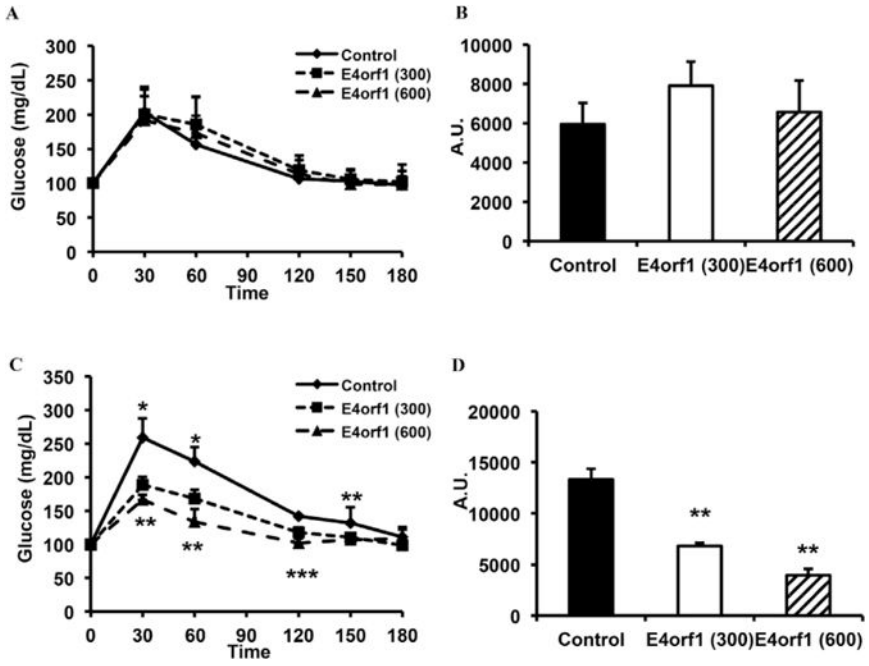

Fig. 4.

Longer duration of high fat diet delays the improvement in GTT induced by E4orf1: Fourteen week old C57BL/6J male mice on 60% fat diet (since 6wk) were inoculated with pBabe-puro (control; black solid lines/bars, n = 6), or with different doses of pBabe expressing E4orf1 (300 μL dose; black dotted lines/bar, n = 6 and 600 μL dose; black big dotted lines/bar, n = 6). E4orf1 expression does not improve blood glucose clearance 1 week p.i. (A–B). A booster dose of the respective vectors 3 week post initial inoculation significantly improved effect of E4orf1 on GTT (C–D). (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$). The bar graphs show area under the curve for respective groups.

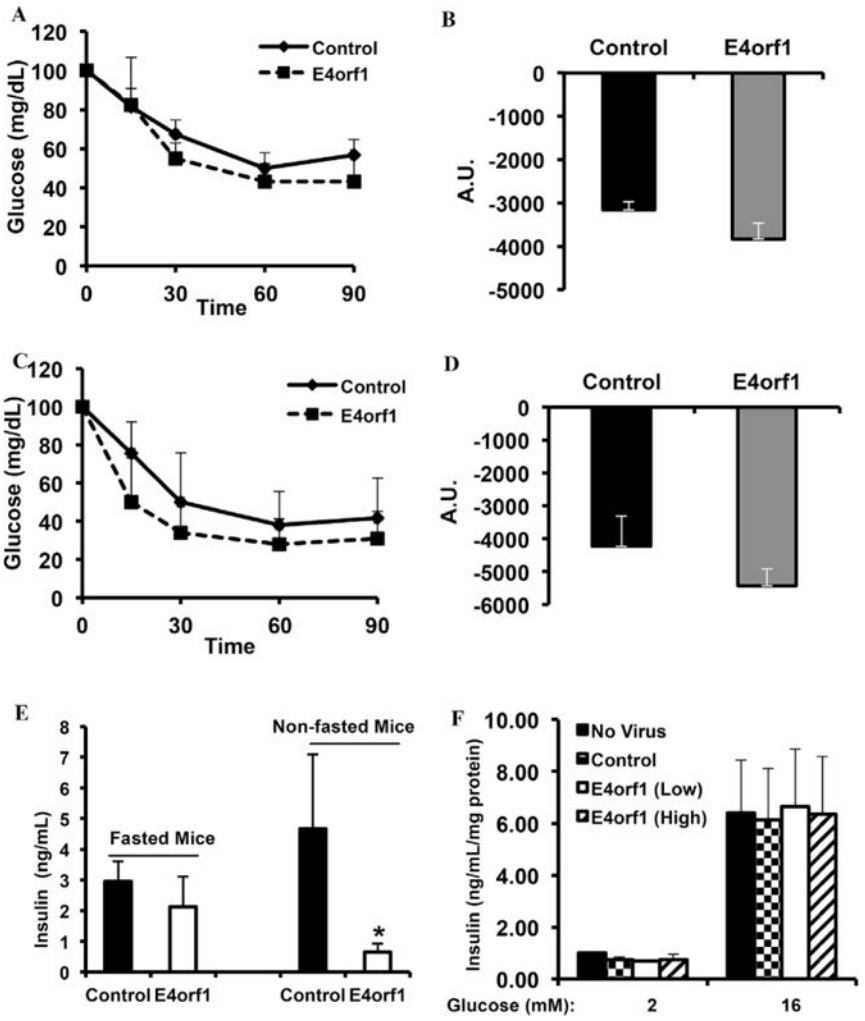

Fig. 5.

E4orf1 improves glucose clearance without increasing insulin sensitivity, production or secretion: Insulin tolerance test (ITT) was performed 1 and 2 week post initial inoculation of control retrovirus (black solid lines, n = 3), or with E4orf1 (black dotted lines; n = 3) in 9 wk old HF fed C57Bl/6j male mice (A–D). The bar graphs (B & D) show area under the curve for respective groups. Serum insulin determined from fasted and non-fasted control (n = 3) and E4orf1 (n ¼ 3) inoculated mice (E). Groups were compared using ANOVA followed by tukey's test (*$p < 0.05$). (F): 832/13 cells were transduced with the indicated viruses overnight. The 832/13 cells were incubated at the designated glucose concentrations for 2 h. Insulin secreted into the medium was measured by ELISA, normalized to total cellular protein, and was expressed as a fold increase relative to insulin secreted at 2 mmol/l glucose in cells that received no virus. Data represent the means ± SE for three independent experiments.

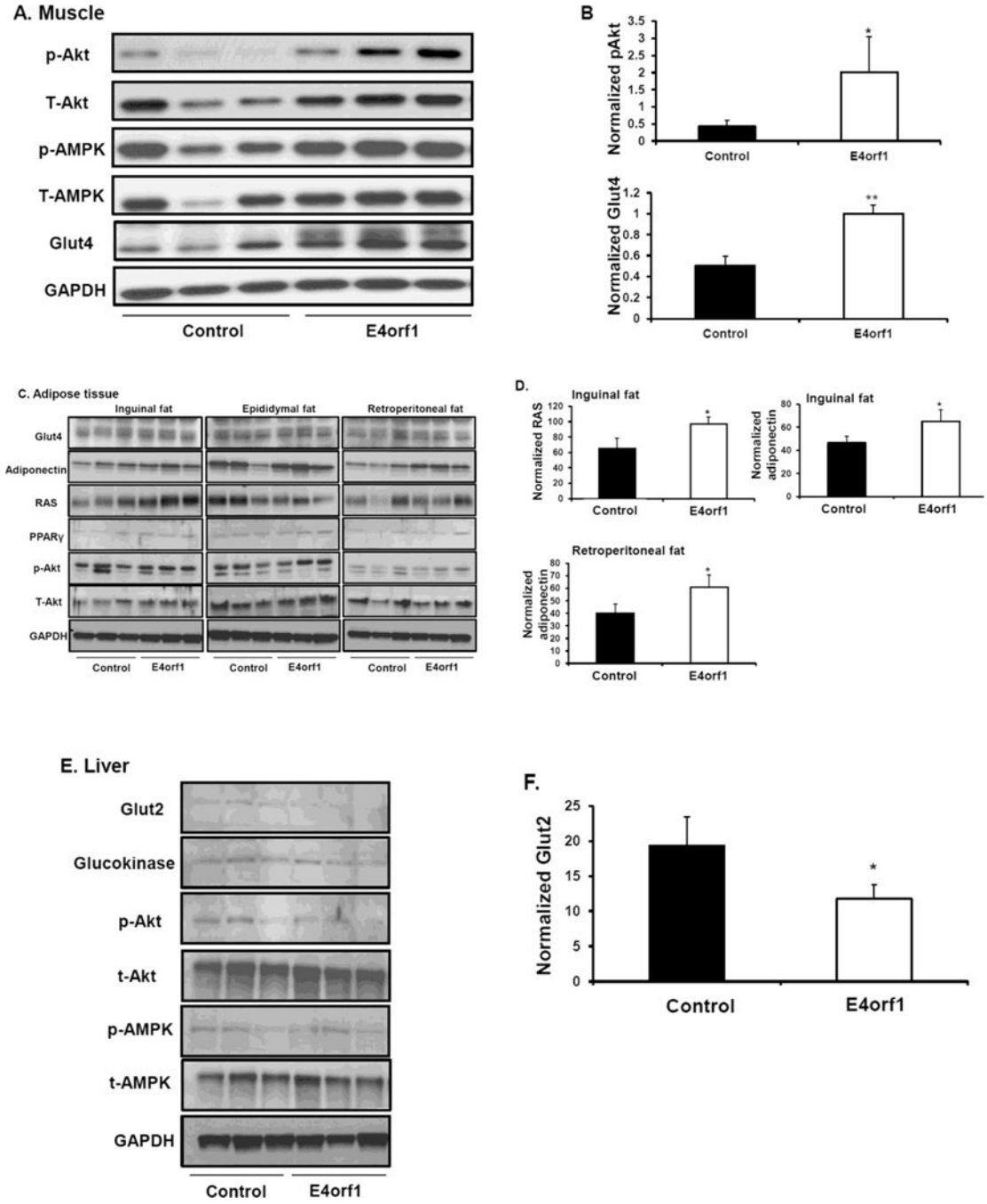

Fig. 6.

E4orf1 enhances cell signaling in glucose disposal pathway: The glucose disposal pathway signaling in muscle, adipose tissue depots, and liver of control (n = 3) and E4orf (n = 3) mice was determined by western blotting. Muscle (A–B): p-Akt, p-AMPK, and Glut4 expressions were normalized to t-Akt, t-AMPK, and GAPDH, respectively. Adipose tissue (C–D): Glut4, adiponectin, RAS, PPARγ, expressions were normalized to GAPDH, p-Akt was normalized to t-Akt. Liver (E–F): Glut2, glucokinase expressions were normalized to GAPDH, p-Akt, p-AMPK were normalized to t-Akt or t-AMPK, respectively. Groups were

compared using ANOVA followed by Tukey's test. Only comparisons that were statistically significant (*$p < 0.05$, **$p < 0.01$) are presented as bar graphs.

Table 1

Pancreatic beta cell mass.

	Tissue area mm^2	Islet area μm^2	Islet fraction %	Avg islet size μm^2
Control (mean)	162.52	1,240,864	0.76	6151.31
Control (SD)	15.60	172,236	0.13	951.42
E4orf1 (mean)	190.39	1,413,198	0.73	5767.87
E4orf1 (SD)	28.30	538,512	0.26	1462.90
TTEST (p value)	0.20	0.62	0.86	0.72

Table 2

Pancreatic alpha and beta cell intensity.

	Total β-cell area	Total β -cell intensity	Mean β -cell intensity	Total α-cell area	Total α-cell intensity	Mean α-cell intensity	Ratio α/β cell area
Control (mean)	251,455	108,000	0.44	32,654	12,208.8	0.37	0.13
Control (SD)	121,331	40,497	0.06	13,905	5107.21	0.003	0.05
E4orf1 (mean)	364,948	141,087	0.38	35,573	13,502.1	0.37	0.10
E4orf1 (SD)	105,153	42,289	0.005	5246	2194.42	0.01	0.03
TTEST (p-value)	0.28	0.38	0.15	0.75	0.70	0.60	0.38